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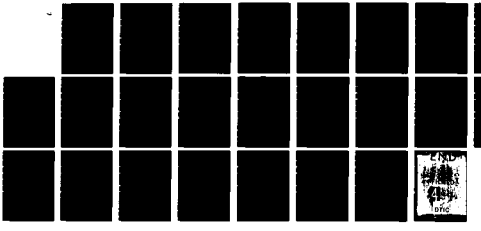

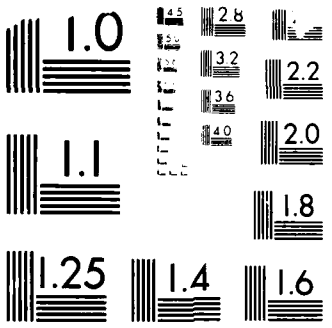

MICROCOPY RESOLUTION TEST CHART

NATIONAL BUREAU OF STANDARDS 1963-A

ANNUAL REPORT ON

THE EFFECT OF CHEMOTHERAPEUTIC AGENTS

ON IMMUNE REACTIONS

Abdul Ghaffar, PhD

August 1982

Supported by the

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND

Fort Detrick, Frederick, Maryland 21701

Contract No. DAMD 17-79-C-9025

University of South Carolina
Columbia, South Carolina 29208

SECURITY CLASSIFICATION OF THIS PAGE *(When Data Entered)*

REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER	2. GOVT ACCESSION NO.
3. RECIPIENT'S CATALOG NUMBER	
4. TITLE *(and Subtitle)* The Effect of Chemotherapeutic Agents on Immune Reactions	5. TYPE OF REPORT & PERIOD COVERED (for the period Annual Sep 81 to Aug 82)
	6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) Ghaffar, Abdul, Ph.D.	8. CONTRACT OR GRANT NUMBER(s) DAMD 17-79-C-9025
9. PERFORMING ORGANIZATION NAME AND ADDRESS University of South Carolina School of Medicine Columbia, South Carolina 29208	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 63750A.3M263750A808.AA.027
11. CONTROLLING OFFICE NAME AND ADDRESS US Army Medical Research and Development Command Fort Detrick, Frederick, MD 21701-ATTN: SGRD-RMS	12. REPORT DATE August 1982
	13. NUMBER OF PAGES 32
14. MONITORING AGENCY NAME & ADDRESS*(if different from Controlling Office)*	15. SECURITY CLASS. *(of this report)* unclassified
	15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT *(of this Report)*

Approved for public release: Distribution unlimited

17. DISTRIBUTION STATEMENT *(of the abstract entered in Block 20, if different from Report)*

18. SUPPLEMENTARY NOTES

19. KEY WORDS *(Continue on reverse side if necessary and identify by block number)*

20. ABSTRACT *(Continue on reverse side if necessary and identify by block number)*

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE

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SUMMARY

Immunotoxic effects of ~~three~~ agents were tested in the animal model for the screening. These agents were: WR 99,210, WR 122,455, and WR-228,258.

WR 99,210 caused a significant enhancement in the phagocytic function (α value), and severe depression in the antibody PFC response when given before antigen, and reduction in the liver and spleen sizes. WR 122,455 and WR 228,258 had similar effects, although with somewhat less severity. Thus, the reduction in the PFC responses and reduction in splenic and hepatic sizes was less pronounced. WR 122,455 was relatively more effective in stimulating the phagocytic function whereas WR 228,258 caused no significant alteration in the phagocytic function. None of the agents seemed to alter significantly the delayed hypersensitivity reaction.

In addition, T and B lymphocyte contents in the peripheral blood of human volunteers treated with WR 171,669 (single dose of 750 to 2000 mg) were enumerated. These studies evaluated 24 individuals before and 2 days after the treatment: half of these volunteers received various doses of the drug and the other half received a placebo. No significant differences were noticed in the proportions of T and B cells in the peripheral blood of these volunteers when placebo and drug-treated groups on Day 0 and Day 2 (pre- and post-treatment) samples were compared.

METHODOLOGY

Mice. Male Balb/C mice weighing 20-25 grams were used in all experiments. Animal experiments were conducted according to the "Guide for the Care and Use of Laboratory Animals" (1972) prepared by the Committee on Revision of the Guide for Laboratory Animal Facilities and Care, Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH)-73-23).

Antigen and Immunization. Sheep erythrocytes (SRBC) suspended in normal saline were used for immunization. Mice received 0.2 ml of 2.5% (1×10^8) SRBC by the i.p. route for studies involving humoral response, whereas 0.1 ml of 0.25% suspension was injected s.c. on the dorsal side of the neck for immunization for the delayed hypersensitivity (DH) reaction. The challenge for elicitation of the DH reaction was given as 0.02 ml of 25% suspension i.d. in the right ear.

Assays

Humoral response. Humoral responses against SRBC were measured 5 days after immunization by assaying spleens for the hemolytic plaque-forming cell (PFC) contents; see Appendix for details (1,2).

DH reactions. A radioisotopic method, described in detail in the Appendix, was used to assess the DH response to SRBC (3).

Phagocytic function. Phagocytic function in mice was measured by the clearance rate of ^{51}Cr-SRBC, as detailed in the Appendix (4).

Solvents and Dosages. WR 99,210 (BJ91326), WR 122,455 (AX76053), or WR 228,258 AG.2HCl (BJ23346.AP-X-56) were administered i.p. as a suspension in 0.2% methylcellulose and 0.4% Tween-80 in saline. The drugs were first

dissolved in methylcellulose-Tween-80 mixture and then diluted to the exact concentration with saline. The desired dose, as recommended by the Walter Reed Army Institute for Research and summarized in Table 1, was injected in 0.4 ml volume. Control mice received 0.4 ml solvent (methylcellulose-Tween-80 in saline). Drugs were injected one day before or one day after the antigen for the antibody PFC or DH test, and two days before the phagocytic assay.

T and B cell enumeration in humans. The T and B cell enumeration was performed on blood samples of human volunteers treated with various doses (ranging from 750 to 2000 mg) of WR 171,669 or placebo by the oral route. The samples were collected on day 0 (pretreatment) and day 2 (post-treatment). The collection of samples was arranged by the Walter Reed Army Institute of Research. The lymphocytes were separated on Ficoll and cryopreserved at Walter Reed by a method recommended by the Principal Investigator (5; see Appendix). T cells were enumerated by using the E-rosette procedure (6) and B cells were assayed by fluorescent staining for surface immunoglobulins (7; see Appendix). Prior to each assay, the viability of cells was determined by the dye exclusion test.

RESULTS

Studies in the Murine Model

It is apparent from Tables 2-4 that all three agents (i.e., WR 99,210, WR 122,455 and WR 228,258) suppressed antibody PFC responses when injected one day before the antigen (SRBC). This suppression was dose-dependent: at the higher dose of each drug, the suppression was generally more marked. The

only exception was the group of mice treated with the higher dose of WR-228,258. Among the three drugs tested, WR 99,210 appears most suppressive. It will also be noticed that suppression was generally more marked at the PFC/spleen level than at the PFC/10^6 level. This is due to markedly significant reduction in the nucleated cell content of spleens in drug-treated groups, particularly those injected with the higher doses. Pretreatment with the lower dose of WR 122,455 did not cause a significant suppression, although there was a trend of reduction in the immune response.

Treatment with drugs one day after the antigen rarely caused a significant suppression. Surprisingly, whenever there was suppression it was more obvious in animals given the lower dose of the drug than in those receiving the higher dose. WR 228,258, in most instances, caused no significant change in the response. In one instance, it actually caused a statistically significant augmentation of the response (IgM per 10^6 in animals receiving 800 mg/kg of the agent). Once again, the reduction in the cellularity of spleens in drug-treated mice was apparent.

The phagocytic function, as measured by the clearance rate of particulate material in mice treated with WR 99,210, was either not altered significantly (as reflected by the K values) or was slightly enhanced (as reflected by the α values). The enhancement was significant ($p < 0.02$) only in mice receiving the higher dose of the drug (Table 5). WR 122,455 consistently augmented the phagocytic function as reflected by both the K value and the α value (Table 6). This elevation of K values was significant in groups treated with the high as well as the low dose of the drug. However, the elevation of α value was statistically significant ($p < 0.005$) only in

mice receiving the lower dose of the drug (Table 6). Treatment with WR-228,258 was without significant effect on the phagocytic function in all instances (Table 7).

WR 99,210 caused an increase in the splenic and hepatic uptake of radiolabeled SRBC, whereas WR 122,455 caused an increase only in the hepatic uptake of the material (Tables 8 and 9). These increases are consistent with the increased clearance of the material from the circulation of these mice and reflect the status of the reticuloendothelial function (4). In mice treated with WR 228,258 there was an increase in the liver uptake but a decrease in the splenic uptake (Table 10).

The spleen weight of animals in none of the drug-treated groups was reduced despite the consistent reduction in their cellularity. In fact, in mice treated with the lower dose of WR 228,258 it was significantly increased. The cause of this increase remains unknown. The liver weights were significantly reduced by doses of WR 99,210 but remained unaffected by the other two agents (Tables 8-10)

None of the three agents tested significantly altered the delayed cutaneous reactions (Tables 11-13).

Studies in Humans

Three groups of individuals were tested. Samples of blood from individuals assigned Alt-1 and Alt-2 were drawn on April 7 and 14, July 7, 14 and 21 (only Alt-1 samples on this date), and August 4, 1980. Blood from 24 volunteers assigned numbers 404-427 were also drawn on the same dates as the pretreatment (day 0) sample. The individuals were treated on day 0 and bled again 2 days later, i.e., on April 9 and 16, July 9, 16 and 23, and August 6, 1980. All tests were performed between March and June, 1982.

It is apparent from data in Table 14 that treatment with placebo or the drug (WR 171,669), regardless of the dose, did not dramatically alter the viability of cells or proportions of T and B lymphocytes. Although it is not possible to apply a statistical test to the data due to small numbers in each group, there was a lack of trend when individuals receiving different doses of the drug were compared.

CONCLUSIONS

The results of studies in the animal model can be summarized as follows. The reticuloendothelial function is either augmented or unaltered. The antibody PFC responses are consistently depressed by all three agents when injected before antigen but are mostly unaltered when injected after the antigen. An overall summation has been presented in Table 15.

These agents provide an interesting contrast from the conventional cytotoxic immunosuppressants which have been categorized in two classes based on their temporal dependence of immunosuppressive effects: Class I and Class II. Class I agents are suppressive when injected before or after the antigen, whereas Class II agents are suppressive only when injected after antigen (8). It appears that the three agents studied here can be classified as neither. There are a number of agents, however, which are immunosuppressive when injected before antigen. These include anti-lymphocyte serum (9), busulphan (8), interferon (10), Corynebacterium parvum (11), and certain other biological products (12).

The mechanism of action of the agents tested in this study is not entirely clear. All of these agents cause lymphocytopenia with unaltered or slightly augmented phagocytic function. The lymphocytopenia may contribute

to the immunosuppression. If this were the sole mechanism of immunosuppression, the relative refractiveness of the immune system to suppressive effects of these agents given after antigen is intriguing and compares with the action of a known immunosuppressive agent, busulphan. It is likely that antigen-activated lymphocytes become resistant to the action of the drug. Alternatively, a defect in the antigen processing/presentation or antigen recognition may contribute to the immunosuppressive effects of these agents. Without further studies, the mechanisms of immunosuppressive action of these agents could not be ascertained.

The assay of T and B lymphocytes in the peripheral blood of volunteers treated with WR 171,669 revealed that this agent did not alter significantly the viability or T cell or B cell proportions in the peripheral blood lymphocytes of human volunteers when tested 2 days after treatment. These results are in concordance with our studies on the effect of this agent on the functional aspects of the immune system in the murine model (13). Although the number of T and B lymphocytes remains normal in individuals receiving WR 171,669, it may not reflect a functional intactness of the cells which can be determined only by performing functional assays.

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Table 1. Doses of drugs administered[a]

Drug	High Dose (mg/kg)	Low Dose (mg/kg)
WR 99,210	64	12.8
WR 122,455	80	16.0
WR 228,258	800	160.0

[a]All drugs were administered in 0.4 ml solvent.

Table 2. Effect of WR 99,210 on anti-SRBC antibody response

Drug Treatment[a] Time	Dose	Cells per Spleen	PFC per[b] 10^6 IgM	PFC per 10^6 IgG	PFC per Spleen IgM	PFC per Spleen IgG
Pre-Ag	Solvent Alone	201	2.902 ± 0.047 (799)	2.860 ± 0.161[c] (724)	5.205 ± 0.050 (160,156)	5.175 ± 0.157[c] (149,594)
	12.8 mg/kg	200	2.295 ± 0.130[d] (197)	1.815 ± 0.307[e,f] (65)	4.548 ± 0.144[d] (35,339)	4.100 ± 0.337[e,f] (12,593)
	64.0 mg/kg	147[f]	1.988 ± 0.163[d] (97)	1.300 ± 0.107[d,e] (20)	4.162 ± 0.169[d] (14,519)	3.396 ± 0.117[d,e] (2,487)
Post-Ag	Solvent Alone	217	2.962 ± 0.040 (916)	2.664 ± 0.140[h] (461)	5.299 ± 0.039 (199,091)	5.001 ± 0.138[h] (100,170)
	12.8 mg/kg	186[g]	2.823 ± 0.041[i] (666)	2.725 ± 0.162[j] (531)	5.096 ± 0.040[d] (124,807)	5.002 ± 0.125[j] (100,560)
	64.0 mg/kg	148[b]	2.876 ± 0.075 (752)	2.574 ± 0.165[h] (375)	5.034 ± 0.106[k] (108,255)	4.722 ± 0.203[h] (52,764)

[a] Drugs injected i.p. 1 day before or after 1 x 10^6 SRBC (Fc measured 5 days after immunization with SRBC.

[b] Log_{10} mean ± 1 s.e. from groups containing a minimum of 16 mice. Numbers in parentheses are geometric means for each group. Data pooled from 2 experiments.

[c] 11/17 gave IgG response

[d] $p < 0.001$

[d] 7/16 gave IgG response

[f] $p < 0.005$ but > 0.001

[g] $p < 0.01$ but > 0.005

[h] 15/17 gave IgG response

[i] $p < 0.025$ but > 0.02

[j] 12/16 gave IgG response

[k] $p < 0.02$ but > 0.01

Table 3. Effect of WR 122,455 on anti-SRBC antibody response

Drug Treatment[a] Time	Dose	Cells per Spleen	PFC per[b] 10^6 IgM	PFC per 10^6 IgG	PFC per Spleen IgM	PFC per Spleen IgG
Pre-Ag	Solvent Alone	209.9	2.913 ± 0.044 (819)	2.763 ± 0.077[c] (579)	5.228 ± 0.059 (168,932)	5.081 ± 0.074[c] (120,464)
	16 mg/kg	197.3	2.749 ± 0.093 (560)	2.676 ± 0.062[d] (474)	5.036 ± 0.110 (108,810)	4.947 ± 0.035[d] (88,572)
	80 mg/kg	157.0	2.241 ± 0.151[e] (174)	1.692 ± 0.147[e,f] (49)	4.551 ± 0.178[g] (35,632)	3.839 ± 0.132[e,f] (6,910)
Post-Ag	Solvent Alone	209.2	3.007 ± 0.129 (1,016)	2.811 ± 0.175[h] (648)	5.332 ± 0.172 (214,540)	5.193 ± 0.188[h] (155,926)
	16 mg/kg	163.6[g]	2.729 ± 0.115[i] (536)	2.765 ± 0.105[j] (582)	4.944 ± 0.125[k] (87,892)	4.975 ± 0.103[j] (94,501)
	80 mg/kg	155.8[i]	2.896 ± 0.112 (788)	2.618 ± 0.155[l] (415)	5.090 ± 0.130 (122,963)	4.862 ± 0.25[illegible] (72,755)

[a] Drugs injected i.p. 1 day before or after 1 x 10^6 SRBC (Fc measured 5 days after immunization with SRBC.

[b] Log_{10} mean ± 1 s.e. from groups containing 9-15 mice. Numbers in parentheses are geometric means for each group. Data pooled from 2-3 experiments.

[c] 10/10 mice gave IgG response [d] 7/10 mice gave IgG response [e] $p < 0.001$

[f] 5/9 mice gave IgG response [g] $p < 0.005$ but > 0.001 [h] 8/15 mice gave IgG response

[i] $p < 0.05$ but > 0.025 [j] 6/15 mice gave IgG response [k] $p < 0.01$ but > 0.005

[l] 7/15 mice gave IgG response

Table 4. Effect of WR 228,258 on anti-SRBC antibody response

Drug Treatment[a] Time	Dose	Cells per Spleen	PFC per[b] 10^6 IgM	PFC per 10^6 IgG	PFC per Spleen IgM	PFC per Spleen IgG
Pre-Ag	Solvent Alone	210	3.041 ± 0.030 (1,098)	3.188 ± 0.059 (1,540)	5.363 ± 0.039 (230,633)	5.510 ± 0.046 (323,479)
	160 mg/kg	182	2.876 ± 0.045[c] (751)	2.830 ± 0.073[d] (676)	5.136 ± 0.036[c] (136,830)	5.088 ± 0.078[e] (122,400)
	800 mg/kg	129[e]	2.970 ± 0.050 (934)	2.603 ± 0.141[d] (401)	5.082 ± 0.080[e] (120,881)	4.717 ± 0.168[e] (52,104)
Post-Ag	Solvent Alone	203	2.912 ± 0.088 (817)	3.044 ± 0.107 (1,107)	5.221 ± 0.097 (166,213)	5.352 ± 0.115 (225,151)
	160 mg/kg	160[f]	2.947 ± 0.030 (884)	3.017 ± 0.079 (1,041)	5.268 ± 0.052 (185,518)	5.238 ± 0.079 (172,985)
	800 mg/kg	122[e]	3.181 ± 0.810[f] (1,516)	3.151 ± 0.102 (1,416)	5.223 ± 0.094 (167,180)	5.226 ± 0.110 (168,357)

[a]Drugs injected i.p. 1 day before or after 1 x 10^6 SRBC (Fc measured 5 days after immunization with SRBC.

[b]Log_{10} mean ± 1 s.e. from groups containing a minimum of 8 mice. Numbers in parentheses are geometric means for each group. Data pooled from 2 experiments.

[c]$p < 0.01$ but > 0.005

[d]$p < 0.005$ but > 0.001

[e]$p < 0.001$

[f]$p < 0.05$ but > 0.025

Table 5. Effect of WR 99,210 on phagocytic function of mice[a]

Treatment	Phagocytic Index[b] 100 x K value	α value
Solvent	6.149 ± 0.314 (9)	6.098 ± 0.125 (9)
12.8 mg/kg	5.833 ± 0.299 (8)	6.692 ± 0.269 (8)
64.0 mg/kg	5.287 ± 0.436 (7)	6.977 ± 0.298[c] (7)

[a]The drug was injected 2 days before test for phagocytic function.

[b]Arithmetic mean ± 1 s.e. Numbers in parentheses are number of mice in each group.

[c]$p < 0.02$ but > 0.01.

Table 6. Effect of WR 122,455 on phagocytic function of mice[a]

Treatment	Phagocytic Index[b] 100 x K value	α value
Solvent	6.149 ± 0.314 (9)	6.098 ± 0.125 (9)
16.0 mg/kg	7.428 ± 0.445[c] (10)	6.926 ± 0.202[d] (10)
80.0 mg/kg	8.032 ± 0.821[c] (9)	6.589 ± 0.211 (9)

[a]The drug was injected 2 days before test for phagocytic function.

[b]Arithmetic mean ± 1 s.e. Numbers in parentheses are number of mice in each group.

[c]$p < 0.05$ but > 0.025.

[d]$p < 0.005$ but > 0.001.

Table 7. Effect of WR 228,258 on phagocytic function of mice[a]

Treatment	Phagocytic Index[b] 100 x K value	σ value
Solvent	6.149 ± 0.310 (9)	6.098 ± 0.125 (9)
160 mg/kg	6.497 ± 0.395 (10)	6.356 ± 0.180 (10)
800 mg/kg	6.007 ± 0.777 (7)	6.031 ± 0.293 (7)

[a]The drug was injected 2 days before test for phagocytic function.

[b]Arithmetic mean ± 1 s.e. Numbers in parentheses are number of mice in each group.

Table 8. Splenic and hepatic changes in mice injected with WR 99,210[a]

Dose	No. Mice per Group	Weight Index[b,d]		^{51}Cr-SRBC Uptake ($\times 10^{-3}$)[c,d]	
		Spleen	Liver	Spleen	Liver
Solvent	9	4.928 ± 0.144	59.595 ± 0.843	60.098 ± 3.810	50.306 ± 1.372
12.8 mg/kg	8	4.739 ± 0.165	53.747 ± 1.975[e]	89.098 ± 5.174[f]	63.778 ± 1.487[f]
64.0 mg/kg	7	4.384 ± 0.256	49.581 ± 1.850[f]	71.590 ± 3.352[g]	61.867 ± 2.063[f]

[a]The drug was injected i.p. 2 days before the i.v. injection of 5×10^8 ^{51}Cr-SRBC. Mice were killed 20 min after SRBC injection and spleens and liver were excised, weighed and assayed for radioactivity.

[b]Spleen or liver weight/whole body weight x 100.

[c]Counts per minute/organ weight.

[d]Arithmetic mean ± 1 s.e.

[e]$p < 0.02$ but > 0.01.

[f]$p < 0.001$.

[g]$p < 0.05$ but > 0.025.

Table 9. Splenic and hepatic changes in mice injected with WR 122,455[a]

Dose	No. Mice per Group	Weight Index[b,d]		^{51}Cr-SRBC Uptake ($\times 10^{-3}$)[c,d]	
		Spleen	Liver	Spleen	Liver
Solvent	9	4.928 ± 0.144	59.595 ± 0.843	60.098 ± 3.810	50.306 ± 1.372
16 mg/kg	10	5.161 ± 0.232	55.699 ± 1.890	51.479 ± 4.057	62.956 ± 3.740[e]
80 mg/kg	9	5.386 ± 0.255	59.700 ± 1.754	66.769 ± 5.071	82.295 ± 2.773[f]

[a]The drug was injected i.p. 2 days before the i.v. injection of 5×10^8 ^{51}Cr-SRBC. Mice were killed 20 min after SRBC injection and spleens and liver were excised, weighed and assayed for radioactivity.

[b]Spleen or liver weight/whole body weight x 100.

[c]Counts per minute/organ weight.

[d]Arithmetic mean ± 1 s.e.

[e]$p < 0.02$ but > 0.01.

[f]$p < 0.001$.

Table 10. Splenic and hepatic changes in mice injected with WR 228,258[a]

Dose	No. Mice per Group	Weight Index[b,d]		^{51}Cr-SRBC Uptake ($\times 10^{-3}$)[c,d]	
		Spleen	Liver	Spleen	Liver
Solvent	9	4.928 ± 0.144	59.595 ± 0.843	60.098 ± 3.810	50.306 ± 1.372
160 mg/kg	10	5.457 ± 0.148[e]	57.707 ± 0.919	64.272 ± 3.304	59.072 ± 2.859[g]
800 mg/kg	7	5.091 ± 0.197	59.137 ± 2.103	46.104 ± 4.040[f]	67.343 ± 3.037[h]

[a]The drug was injected i.p. 2 days before the i.v. injection of 5×10^8 ^{51}Cr-SRBC. Mice were killed 20 min after SRBC injection and spleens and liver were excised, weighed and assayed for radioactivity.

[b]Spleen or liver weight/whole body weight x 100.

[c]Counts per minute/organ weight.

[d]Arithmetic mean ± 1 s.e.

[e]$p < 0.025$ but > 0.02.

[f]$p < 0.05$ but > 0.025.

[g]$p < 0.02$ but > 0.01.

[h]$p < 0.001$.

Table 11. Effect of WR 99,210 on anti-SRBC DH reaction in mice[a]

Drug Treatment Time	Dose	DH Reaction Relative Ear Reaction[b]
Pre-Ag	Solvent	1.362 ± 0.103
	12.8 mg/kg	1.439 ± 0.090
	64.0 mg/kg	1.238 ± 0.083
Post-Ag	Solvent	1.488 ± 0.084
	12.8 mg/kg	1.235 ± 0.101
	64.0 mg/kg	1.271 ± 0.064

[a]The drug was injected i.p. 1 day before or 1 day after s.c. immunization with 5×10^6 SRBC. Mice were challenged intradermally 6 days later with 1×10^8 SRBC in the right ear and assayed the following day.

[b]Arithmetic mean of ratios for groups of 10-11 mice ± 1 s.e. calculated as follows: $\frac{\text{cpm in challenged ear}}{\text{cpm in control ear}}$.

Table 12. Effect of WR 122,455 on anti-SRBC DH reaction in mice[a]

Drug Treatment Time	Dose	DH Reaction Relative Ear Reaction[b]
Pre-Ag	Solvent	1.362 ± 0.103
	16 mg/kg	1.347 ± 0.134
	80 mg/kg	1.438 ± 0.090
Post-Ag	Solvent	1.488 ± 0.084
	16 mg/kg	1.774 ± 0.167
	80 mg/kg	1.612 ± 0.146

[a]The drug was injected i.p. 1 day before or 1 day after s.c. immunization with 5×10^6 SRBC. Mice were challenged intradermally 6 days later with 1×10^8 SRBC in the right ear and assayed the following day.

[b]Arithmetic mean of ratios for groups of 10-11 mice ± 1 s.e. calculated as follows: $\frac{\text{cpm in challenged ear}}{\text{cpm in control ear}}$.

Table 13. Effect of WR 228,258 on anti-SRBC DH reaction in mice[a]

Drug Treatment Time	Dose	DH Reaction Relative Ear Reaction[b]
Pre-Ag	Solvent	1.362 ± 0.102
	160 mg/kg	1.200 ± 0.096
	800 mg/kg	1.205 ± 0.140
Post-Ag	Solvent	1.488 ± 0.084
	160 mg/kg	1.470 ± 0.095
	800 mg/kg	1.602 ± 0.132

[a]The drug was injected i.p. 1 day before or 1 day after s.c. immunization with 5×10^6 SRBC. Mice were challenged intradermally 6 days later with 1×10^8 SRBC in the right ear and assayed the following day.

[b]Arithmetic mean of ratios for groups of 10-11 mice ± 1 s.e. calculated as follows: $\frac{\text{cpm in challenged ear}}{\text{cpm in control ear}}$.

Table 14. T and B cell proportions in volunteers treated with WR 171,669

Group & Dose	Code No.	Drug or Placebo[a]	% Viable Cells Recovered		% T Lymphocytes		% B Lymphocytes	
			Day 0	Day 2	Day 0	Day 2	Day 0	Day 2
Alt[b]	Alt-1, 4/7	None	83		41.0		20.0	
	Alt-2, 4/7		91		66.0		17.0	
	Alt-1, 4/14		75		48.0		19.9	
	Alt-2, 4/14		77		41.8		16.8	
	Alt-1, 7/7		80		40.0		15.9	
	Alt-2, 7/7		74		37.0		26.0	
	Alt-1, 7/14		71		30.0		18.5	
	Alt-2, 7/14		65		23.0		18.2	
	Alt-1, 7/21		86		39.0		15.5	
	Alt-1, 8/4		90		47.0		12.8	
	Alt-2, 8/4		88		50.0		13.2	
II	404	Drug	84.0	85.0	51.0	41.0	19.3	21.1
750 mg	405	Placebo	88.0	92.0	54.0	38.0	12.4	15.0
	406	Placebo	90.0	89.0	47.0	33.0	18.1	18.4
	407	Drug	87.0	89.0	35.0	31.0	20.6	17.4
III	408	Drug	90.0	88.0	59.0	52.0	16.7	15.5
1000 mg	409	Placebo	81.0	87.0	45.0	43.0	18.2	18.6
	410	Placebo	84.0	78.0	54.0	54.0	21.0	24.9
	411	Drug	86.0	88.0	47.0	48.0	14.2	13.2

Table 14 (continued)

Group & Dose	Code No.	Drug or Placebo	% Viable Cells Recovered		% T Lymphocytes		% B Lymphocytes	
			Day 0	Day 2	Day 0	Day 2	Day 0	Day 2
IV	412	Drug	85.0	84.0	49.0	55.0	20.0	19.5
1250 mg	413	Placebo	88.0	85.0	59.0	58.0	15.0	14.4
	414	Drug	86.0	79.0	46.0	50.0	19.2	27.0
	415	Placebo	87.0	91.0	50.0	53.0	16.9	17.4
V	416	Drug	69.0	86.0	39.0	56.0	15.6	18.4
1500 mg	417	Drug	70.0	74.0	11.0	47.0	22.6	14.4
	418	Placebo	77.0	81.0	22.0	49.0	23.5	11.3
	419	Placebo	72.0	76.0	40.0	38.0	15.3	12.3
VI	420	Drug	84.0	89.0	34.0	46.0	11.6	13.5
1750 mg	421	Placebo	NT[c]	91.0	NT	51.0	NT	9.3
	422	Placebo	80.0	88.0	48.0	51.0	11.5	9.3
	423	Drug	81.0	84.0	21.0	48.0	9.5	8.5
VII	424	Drug	86.0	87.0	44.0	50.0	9.8	8.4
2000 mg	425	Placebo	88.0	84.0	59.0	48.0	13.5	10.8
	426	Drug	93.0	89.0	51.0	45.0	8.7	10.3
	427	Placebo	84.0	88.0	54.0	59.0	6.5	5.4

[a]Single oral dose. [b]Did not receive any drug or placebo. Only day 0 sample was drawn.
[c]NT, not tested; the sample did not yield adequate numbers of viable cells.

Table 15. Summary of the effects of various anti-malarial agents on immune functions in mice

Drug	Antibody PFC[a]				DH Reaction		Organ Wt/Cellularity			Phagocytic Activity	
	IgM		IgG				Spleen	Spleen	Liver		
	Pre-Ag	Post-Ag	Pre-Ag	Post-Ag	Pre-Ag	Post-Ag	Cellularity	Weight	Weight	K	α
99,201	↓↓↓	↓	↓↓↓	—	—	—	↓↓	—	↓↓	—	↑
122,455	↓↓	↓	↓↓	—	—	—	↓	—	—	↑↑	↑
228,258	↓↓	—	↓↓	—	—	—	↓↓	↑	—	—	—

[a]Drug injected one day before (Pre-Ag) or one day after (Post-Ag) the injection of SRBC.

APPENDIX

Plaque-forming cell (PFC) assay.[1,2] Five days following i.p. immunization of mice with sheep red blood cells (SRBC), spleens were excised and gently disrupted by means of a glass/Teflon tissue homogenizer in Hank's balanced salt solution containing 0.1% bovine serum albumin (BSA-HBSS). The cells were washed once and resuspended in 4 ml of the same medium. The tubes were allowed to stand for 60 sec to permit clumps and tissue debris to settle. A 1 ml aliquot of singly-dispersed cells was removed from the top of the suspension and transferred to another tube. The number of nucleated cells per ml of this suspension was enumerated and adjusted to a concentration of 2-3 x 10^6 nucleated cells per ml. One volume (0.1 ml) of this suspension was mixed with one volume of 10% SRBC suspension, one volume of medium and one volume of 1:2 dilution of complement. Aliquots (0.1 ml) of the mixture were placed in chambers prepared by assembling two 3" x 1" microscopic slides by means of 5 mm wide double-faced adhesive tape. The chambers were sealed along the edges with molten wax and incubated at 37°C in a humidified incubator for 40 min, after which the number of plaques was enumerated. This technique allowed the assay of the IgM PFC contents of the splenic suspension. For the detection of IgG PFC, the splenic cell suspension was mixed with equal volumes of 10% SRBC, rabbit anti-mouse IgG serum at an optimal dilution (previously determined) and complement. The mixture was placed in chambers and incubated as above. Plaques observed in these chambers were a mixture of IgM and IgG, and the latter were derived by the deduction of the IgM PFC calculated from plaques

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1. Cunningham and Szenberg. Immunology 14:599, 1968.
2. Dresser and Wortis. In Handbook of Experimental Immunology (D. M. Weir, ed.), p. 1054. Blackwell Scientific Publications, Oxford, 1967.

observed in chambers containing no antiserum against mouse IgG. From the number of plaques per chamber was calculated the number of PFC per 10^6 nucleated splenocytes and also PFC per spleen.

Delayed hypersensitivity (DH) reaction.[3] Mice were injected s.c. with 5×10^6 SRBC in 0.1 ml volume on the dorsal side of the neck. Six days later they were challenged with 1×10^8 SRBC in 20 µl volume i.d. in the pinnea of the ear; 24 hr later 2 µCi of ^{125}IUdR in 0.2 ml volume was injected i.v. via the tail vein. Both ears were excised 18 hr later at the base of the pinnea and radioactivity incorporated in each ear was measured in a gamma spectrometer. The ratio of radioactivity incorporated in injected and non-injected ears was calculated and was taken as an index of the DH reaction. This method has been reported as a reliable procedure for assaying the DH reaction to a number of agents and we have confirmed the validity of the technique in our own laboratory.

Phagocytic function of the reticuloendothelial system (RES).[4] SRBC were washed three times in saline and to 1 ml of packed SRBC was added 100 µCi of ^{51}Cr (but always less than 5 µg Na_2CrO_4). The cells were mixed and incubated for 30 min at room temperature and then washed three times in saline. Washed cells were suspended at a final concentration of 10% (v/v) in saline and 0.2 ml of this suspension (4×10^8 SRBC) was injected i.v. via the tail vein and 20 µl of blood was collected immediately (zero time sample) from the retro-orbital sinus using a heparinized capillary (hemato-

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3. Vadas et al. Int. Arch. Allergy Appl. Immunol. 49:670, 1975.
4. Sljivic and Warr. In Manual of Macrophage Methodology (H. B. Herscowitz et al., eds.), p. 447. Marcel Dekker, New York, 1981.

crit) tube. The same volumes of samples were collected similarly at 2, 5, 10 and 15 min intervals from the time of original bleeding. All samples were placed in test tubes and the radioactivity was counted in a gamma scintillation spectrometer. The radioactivity in the sample represented the relative concentration of SRBC in circulation at a particular time. The cpm were converted to log and plotted against time, and the slope of the best-fitting straight line for the plot was calculated; it represented the phagocytic index K. Since the concentration of particles in circulation, and hence the K value, is dependent on the total body weight of the mouse, its liver and spleen weights, a corrected phagocytic index α was calculated as follows:

$$\alpha = \frac{w}{l + s} \sqrt[3]{K}$$

where w is whole body weight, l is liver weight and s is spleen weight.

Localization of particulate material in liver and spleen. Spleen and liver were removed intact and weighed. The radioactivity associated with each organ (proportionate with their SRBC content) was measured in a gamma counter and the radioactivity was calculated per gram of the organ weight.

Presentation of data. Numbers of spleen cells have been presented as geometric means. PFC per 10^6 splenocytes and per spleen have been recorded as $\log_{10}$ mean together with one standard error. Anti-log of $\log_{10}$ mean are recorded in parentheses. DH reaction has been reported as the mean of relative ear reaction together with the standard error of the mean. The relative ear reaction was calculated as follows:

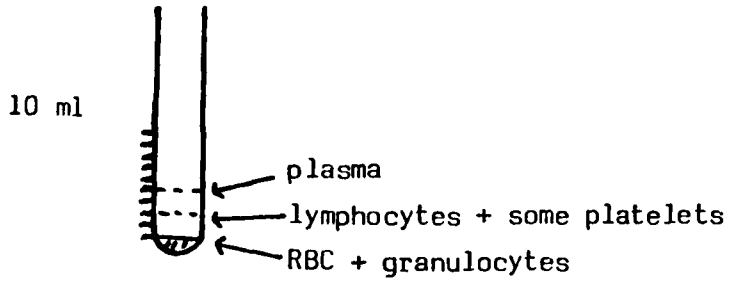
$$\frac{^{125}\text{IUdR incorporation (cpm) in challenged ear}}{^{125}\text{IUdR incorporation (cpm) in control ear}}$$

Data on phagocytic function have been expressed as the mean of K or α values within limits of one standard error. The uptake of SRBC in spleen and liver is expressed as the mean of cpm per gram of tissue within one standard error of the mean.

Statistical significance of the data was evaluated by the standard two-tailed Student's t-test with correction for small groups and expressed as p values. P values greater than 0.05 were considered not significant.

Preparation of human lymphocytes (method recommended to Walter Reed Army Institute of Research):

1. Collect 20 ml heparinized blood. Spin at 18 x g (300 rpm) in a bench centrifuge for 15 min. Remove platelet-rich (top 3/4 volume) plasma and discard, leaving about 1/4 of the supernatant in the tube.
2. Spin remainder at 600 x g for 5 min.
3. Discard all but 1 ml of plasma. Harvest this volume of plasma, the buffy coat and 1 ml of red cells. Mix the harvested cells and layer on top of lymphocyte separation medium (LSM; Litton Bionetics). A 2 ml blood sample can be layered on 3 ml LSM.
4. Centrifuge at 400 x g for 20 min. The resultant tube should look like this:



5. Pipet plasma off down to 1/16 inch from the lymphocyte band and discard.
6. Collect all the contents of the lymphocyte band. Dilute with medium (2 ml of LSM-lymphocytes + 8 ml medium). Spin at 600 x g for 5 min. Decant the supernatant. Resuspend cells in RPMI-1640 complete medium (containing 20% fetal calf serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine).

7. Wash the cells 3X in 1 ml complete medium, resuspend, count the cell concentration and adjust at 12-15 x 10^6 cells/ml.

Cryopreservation of human lymphocytes (method recommended to Walter Reed Army Institute of Research):

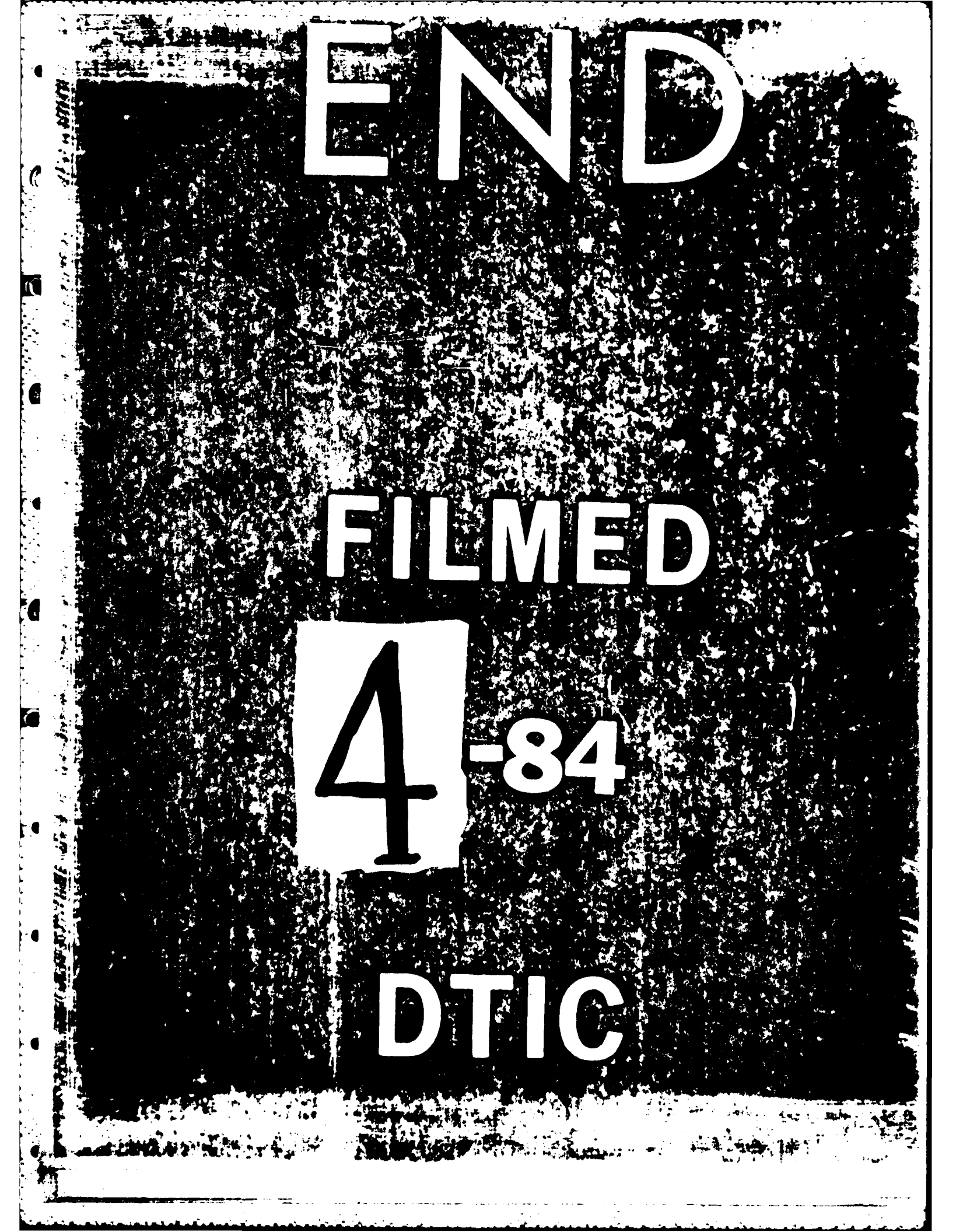
1. Divide the lymphocyte suspension into two aliquots in labeled screw-top polypropylene 2 ml tubes and chill to 4°C.
2. Cool the freezing chamber to 4°C.
3. Add an equal amount of cryopectant (20 ml DMSO + 80 ml complete medium) to the cell suspension.
4. Place samples in the cryopreservation chember and start cooling at 1° per min to -40°C, then rapidly cool to -100°C.
5. Samples must be stored in liquid nitrogen and transported on dry ice.

Determination of the viability of cells in human samples. Samples prepared, frozen and shipped by the Walter Reed Army Institute for Research were removed from the liquid nitrogen and thawed in a 37°C water bath on the day of test. As soon as the specimen was thawed, it was diluted 10:1 with RPMI-1640 medium containing 20% fetal calf serum. The dilution was conducted at room temperature by adding the medium drop-by-drop and with continuous gentle mixing (swirling) of the sample. The cells were washed and resuspended in 1 ml medium. A small aliquot was mixed with an equal volume of 0.1% trypan blue and the viable cells were counted on the basis of the dye exclusion.

Enumeration of human T lymphocytes. Sheep erythrocytes (SRBC) obtained from Flow Laboratories as heparinized blood were washed and suspended in RPMI-1640 medium at a concentration of 1%. Neuraminidase (Sigma) was diluted to 1 U/ml and 0.4 ml of this solution was added to and mixed with 10 ml of 1% SRBC and incubated at 35°C for 35 min. The neuraminidase-treated SRBC were washed 3 times and resuspended in the medium at a concentration of 0.5%. Lymphocytes were adjusted to a concentration of 5 x 10^6 cells/ml and mixed with an equal

volume (0.1 ml) of the neuraminidase-treated SRBC suspension. The mixture was incubated at 37°C for 5 min, centrifuged at 400 g for 5 min, and the tubes were placed in an ice bath for 45 min. The pellet was gently resuspended and rosettes were counted in a sample of at least 200 cells. Lymphocytes with 3 or more bound SRBC were considered a rosette.

Enumeration of human B lymphocytes. Lymphocytes were suspended at 5×10^6 cells/ml in Hepes-buffered RPMI-1640 medium containing 0.02% sodium azide. One-tenth ml of lymphocyte suspension was mixed with 0.1 ml of aggregated normal rabbit IgG and incubated on ice for 10 min. One-tenth ml of FITC-labeled rabbit anti-human immunoglobulin (at a previously determined optimal dilution) was added to the tube and the incubation at 4°C was continued for another 30 min. The cells were washed three times, resuspended in 0.2 ml azide-containing medium, and the fluorescent cells were counted.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